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Abstract

Tall fescue (*Festuca arundinacea* Schreb.), the most important pasture grass in Arkansas, exhibits different agricultural properties when it is infected by its mutualistic endophyte *Acremonium coenophialum* Morgan-Jones and Gams. We postulate that the presence of endophyte exerts a stress on the host that enhances or detracts from the host's ability to express specific genes. We tested this hypothesis by heat stressing infected and non-infected, juvenile and mature tall fescue, and examining their protein profiles by SDS-PAGE analysis. The results indicate that mature, infected, stressed grass produced greater amounts of Rubisco (ribulose bisphosphate carboxylase-oxygenase) than all other treatments. Additionally, the mature, infected, stressed grass exhibited a 20 kDalton protein band which was not apparent in other treatments. These observations support the possibility that the endophyte prestresses the grass, and they suggest a molecular mechanism for this response.

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Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is an important and well-adapted forage and turf grass in Arkansas, as well as in many other regions of the world. Certain cultivars of this species are known to harbor the mutualistic endophyte *Acremonium coenophialum* Morgan-Jones and Gams. The association seems to contribute many selective advantages to the host plant including resistance to insect herbivory (Patterson et al., 1991), disease (Gwinn and Gavin, 1992), nematodes (West et al., 1989) increased photosynthetic output (Clay, 1990) and an increased tolerance to drought (West et al., 1993) and heat (Clay, 1990). Unfortunately, toxic alkaloids produced by the fungus promote the conditions known as "ryegrass staggers" and "fescue toxicosis" in grazing animals, conditions that cause an estimated annual loss of $50 million (1989 figures) in Arkansas alone (Daniels, 1989).

Insect and nematode resistance in endophyte-infected (E+) plants has been fairly well-characterized (Latch, 1993). Proposals have been made to account for the observed increase in photosynthetic output (Clay, 1990). Studies that have been made to suggest mechanisms responsible for the observed increase in the stress survival of E+ over non-infected (E-) grasses suggest a possible combination of morphological traits present in infected grasses (Arachavaleta et al., 1989) with induced differences in carbohydrate metabolism (Richardson et al., 1992, 1993). It is our hypothesis that the presence of the endophyte in tall fescue induces a mild "pre-stress" on the plant, mobilizing the plant's endogenous defense systems that better enable it to survive greater stresses induced by drought or heat. To test this idea, we made preliminary studies examining differences in the SDS-PAGE electrophoresis of the total proteins of an E+ (cultivar "Kentucky-31") and E- variety (cultivar "Fawn") before and after a severe heat stress. These tests were further subdivided to examine differences between juvenile (<60 days post germination) and mature (>6 mon) grasses.

Materials and Methods

Six 36 cm x 26 cm plots each of E+ and E- tall fescue were seeded using seed samples obtained from the Arkansas Plant Board. E+ samples were *F. arundinacea* Schreb. var. *genuina* cultivar "Kentucky-31" and E- samples were of the cultivar "Fawn" (same variety). The samples were Plant Board certified at 99% and 0% *Acremonium coenophialum* infection, respectively. Both cultivars used were hexaploids (2n = 42). Infection was recertified using protocol outlined by the Association of Official Seed Analysts. The plots were randomized and maintained for 8 months (June 1992 - March 1993) in a greenhouse. Seedings were thinned to the strongest plants every three days from 2 weeks to 4 weeks post-germination. Plots were fertilized once monthly with 50-30-15 fertilizer (Super K-Gro). Of these randomized plots, mature samples were selected by choosing one each of the healthiest plots for analysis. The chosen plots were moved to a controlled environmental

chamber (Baxter Scientific Products Cryo-Fridge) at 24°C and 12 h/day light exposure for one week prior to removing 5 g leaf tissue for pre-stress analysis. Juvenile samples were grown from germination in the chamber and were sampled at the 30 day post-germination mark for pre-stress analysis (juvenile and mature plants were sampled simultaneously), removing only 1-2 g of tissue due to lowered availability. The plots were then subjected to heat stress (42° for 48 h with 12 h/day light) and were then sampled post-stress. No readjustment period was allowed between stress and plant harvest.

Protein was extracted immediately after harvest by first freezing the sample tissue with liquid nitrogen and grinding with porcelain mortar and pestle. The ground tissue was homogenized into 1.5x volume extraction buffer (modified from Mehta et al., 1991, leupeptin excluded). The homogenate was strained through Miracloth and was centrifuged at 12,000 x g for 20 min to remove cell debris. Total proteins were then separated by SDS-PAGE in both 12% and in 10-12% continuous gradient gels (Sambrook et al., 1989). Proteins were stained with Coomassie Blue and lanes were analyzed for band differences on a Molecular Dynamics Model 300A laser densitometer and analyzed using ImageQuant Analysis Program (Molecular Dynamics). Adjustment for protein concentration between extractions was made by equalizing the lowest density pixels between the lanes of the imaged gels.

Results

In general, each preparation yielded many distinct bands on the SDS-PAGE gels. Although the bands were more easily visualized in the mature tissues, they were apparent to the laser scanner in the juvenile preparations as well. The Rubisco (ribulose bisphosphate carboxylase-oxygenase) large subunit band was clearly visible in all preparations and was migrating at the expected distance for its 53 kDa weight. When considering the juvenile samples only, we found no differences between any treatment or between any samples for any protein band (Fig. 1).

By using the band quantification mode of the ImageQuant Analysis Program, we found five distinct and significant protein peaks in the mature stressed E+ sample, while only four peaks were found in either mature stressed E- or mature non-stressed E+ samples (Table 1). Although the high molecular weight peaks may represent different proteins (peaks 1; Table 1), the discovery of a unique peak in the stressed grass is of current interest. When compared to either mature stressed E- or the mature non-stressed E+ samples, the additional peak was in the 20 kDa range of molecular weights, weight range being determined by migration of known molecular weight markers. Curiously, each of the other four peaks was apparent in the mature stressed E- and mature non-stressed E+ samples with the only difference in magnitude being the Rubisco.

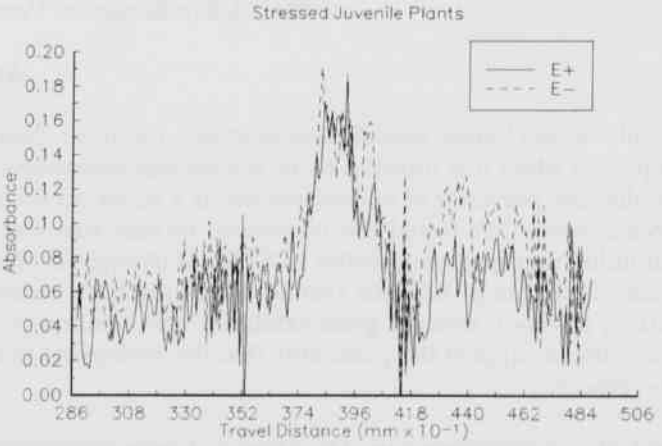

Fig. 1. Profiles of electrophoretic separations of proteins from stressed juvenile E+ and E- plants. The rubisco peak is centered at approximately 380 x 10^{-1} mm.

Table 1. Quantified peaks in stressed mature E+ and E- plants. Peak numbers refer to approximately corresponding peaks in E+ and E- plants.

Infection status	Peak	Peak Y-position
E+	1	156
	2	391
	3	454
	4	516*
	5	716
E-	1	251
	2	396
	3	434
	5	781

*lacks corresponding peak in E- scan

Because Coomassie Blue stains different proteins to differing extents, quantitative determinations of proteins without standard curves for the protein in question are not valid. However, comparisions of relative amounts between bands of the same protein (particularly those on a single gel) are allowable, as are, to a lesser extent, comparisons between different proteins on the same gel (Hames, 1990). Given this proviso, Rubisco was evident in

what appeared to be a higher concentration than other proteins. We observed approximately a 5-fold increase in the Rubisco band intensity in the mature stressed E+ samples as compared to all other mature samples (Fig. 2). The magnitude of this protein was approximately the same (about 6% of total protein) in mature stressed E- and mature non-stressed E+ samples.

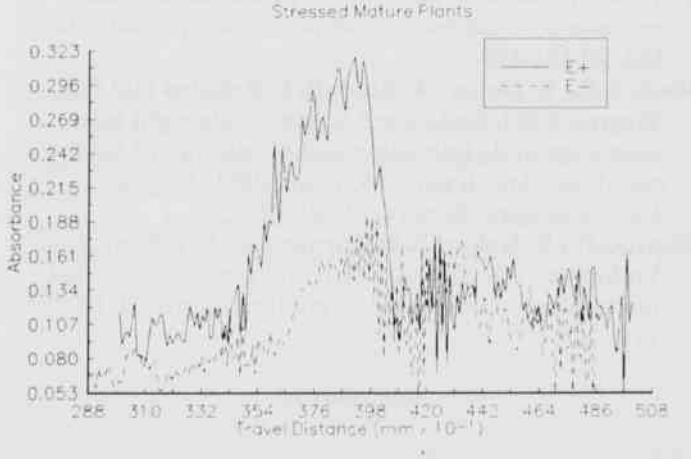

Fig. 2. Profiles of electrophoretic separations of proteins from stressed mature E+ and E- plants. The difference in peak absorbance at 380 x 10^{-1} mm reflects a five-fold increase of Rubisco in the stressed E+ sample.

Discussion

Our working hypothesis involves the notion that the endophyte induces some type of stress related response in the grass host. Futhermore, because other studies have demonstrated increased drought tolerance, greater growth, and osmotic differences (Clay, 1990) between E+ and E- plants, our hypothesis suggests that specific protein synthesis pathways are being regulated differentially. If that is the case, SDS-PAGE analysis should detect specific protein differences between infected and non-infected grass. In the presence of an environmental stress, that effect should be magnified and more easily observed.

The preliminary observations presented in this work support the "pre-stress" hypothesis; however, they do not eliminate alternative hypotheses. Specifically, we observed no protein differences among juvenile samples regardless of treatment (Fig.1). Because we and others (Welty et al., 1986) have not been able to reliably observe fungal hyphae in leaves younger than 30 days, we suspect that the influence of the endophyte is non-existent. Our observation of no protein differences among juvenile tissues subjected to either stress or infection agrees well with the observed life cycle of the endophyte and the above prediction.

When we considered the mature tissue, we made two major classes of observations. First, we observed a unique protein peak in the stressed E+ tissue. The approximate molecular weight (20 kDaltons) of this peak coincides well with a known superfamily of low molecular weight heat shock proteins (LMW HSP's) in plants, ranging in weight from 17 kDa to 27 kDa. These proteins are present in negligible amount in unstressed tissue, but can be some of the most abundant proteins present in heat stressed tissue (Vierling, 1991). The fact that eukaryotes other than plants possess far fewer of these LMW HSP's eliminates the possibility of the band difference being directly attributable to the presence of the endophyte and indicates that it is most likely the product of the infected plant itself. While the absence of the band in unstressed E+ seems to preclude the "pre-stress" hypothesis, it should be noted that these proteins, if indeed the band is a member of this family, seem to be largely heat regulated (Vierling, 1991). The fact that E+ plants were able to mobilize production of this HSP would support the "pre-stress" hypothesis, but as this protein difference has not yet been characterized, this would not preclude other hypotheses.

Finally, we observed that the stressed E+ tissue produced more Rubisco than either the stressed E- or non-stressed E+ material. As Rubisco is the primary enzyme system involved in photosynthesis, this would be consistent with enhanced photosynthetic rates and osmotic balance previously observed in E+ material (Clay,1990). Also, as the apparent levels of Rubisco were similar in stressed E- and non-stressed E+ (both slightly greater than in non-stressed E-), this suggests that E+ is normally producing this protein at a similar rate as stressed E- tissue.

In conclusion, we have preliminary evidence from three sources to suggest that the presence of endophyte in tall fescue induces some type(s) of pre-stress condition(s) in the plant. First, we only observed an effect in mature plants. As the endophyte can not be detected until after 30 days post-germination, this observation supports the hypothesis. Second, an additional protein peak in the 20 kDalton molecular weight range was observed in stressed E+ grass. These proteins are in the molecular weight range of known heat shock proteins particular to plants, providing a possible molecular mechanism for the enhanced heat and drought resistance observed in E+ tall fescue. Finally, preliminary evidence suggests that stressed E+ material produced quantitatively more Rubisco than either stressed E- or non-stressed E+ tissue, while stressed E- and non-stressed E+ tissue produced similar amounts; either of which is more than non-stressed E- material. As these results are preliminary, additional research involving other genotypes must be conducted.

Additionally, other stress responses must be eliminated to ascertain that we are observing a "pre-stress" induced by endophyte.

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